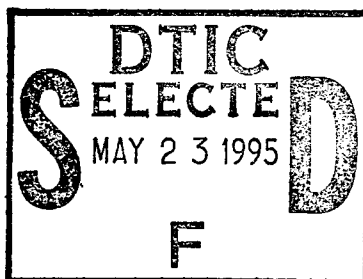# ANIMAL TEST ALTERNATIVES

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Munitions Cytotoxicities In Vitro

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Rapid, inexpensive, and reliable methods are needed for the toxicological screening of chemical substances with the potential to affect health and the environment. One such method, the neutral red (NR) cytotoxicity assay, is based on incorporation of the supravital dye neutral red into lysosomes of viable cells. The NR uptake assay can be used to detect cytotoxic or cytostatic effects of chemical substances capable of damaging cells, has been adapted to microtiter tissue culture systems, and can be analyzed by means of automated spectrophotometric microplate readers [1,2]. Neutral red cytotoxicity assays in continuous rat hepatoma H4IIE cells have been applied to a variety of environmentally important munitions and related compounds. The H4IIE cells maintain inducible oxidative microsomal enzymes and were chosen because of their application to detecting other xenobiotics in environmental and biological specimens [6].

MATERIALS AND METHODS

The H4IIE rat hepatoma cells were obtained from the American Type Culture Collection, Rockville, Maryland, and were grown at 37°C in a 5% CO_2 atmosphere. Medium was Eagle's minimal essential medium (MEM) with

Earle's salts supplemented with 10% fetal bovine serum, 10% newborn calf serum, 10 mM nonessential amino acids, and 50 μg/ml gentamicin. The NR uptake assays were conducted in the same medium, except that gentamicin was raised to 250 μg/ml to accommodate nonsterile munitions. Dimethyl sulfoxide (DMSO) was the solvent for all test chemicals and was maintained at 0.25% (v/v) in NR uptake assays.

Neutral Red Cytotoxicity Assays [2]

Day 1. Monolayers were trypsinized, dispersed, and 96-well microtiter plates were seeded at 9×10^{-3} cells per well in 0.2 ml of fresh medium.

Day 2. Medium was aspirated, and 0.2 ml of fresh medium, containing the desired test chemical concentration, was added to each of eight replicate wells for each toxicant concentration.

Day 3. Medium was aspirated from all wells exposed to chemicals and solvent controls and replaced with 0.2 ml of medium containing NR (0.005%, w/v) for 4 hr at 37°C. The NR-staining solution was removed and cells were washed 2 min with a fixative (1% $CaCl_2$, 1% formaldehyde). After aspiration of the fixative solution, the dye remaining in the cells was extracted with 0.2 ml 1% acetic acid, 10% butanol, and 50% ethanol for 1.25 hr. Microtiter plates were agitated 2 min to distribute the dye, and optical absorbance measurements were made by means of an automatic microtiter plate reader.

Measurements

Values of NR uptake in multiple wells for each toxicant dilution were plotted as mean percentages ($\pm 1\sigma$) of controls exposed only to DMSO. Midpoint cytotoxicity values (NR_{50}) are defined as that amount of toxicant that would reduce NR uptake to 50% of its control value, based on regression curves of the uptake data in the region of change.

Munitions Abbreviations

2,4-Dinitrotoluene (2,4-DNT); 2,6-dinitrotoluene (2,6-DNT); 1,3-dinitrobenzene (1,3-DNB); 2-amino-4-nitrotoluene (2A-4-NT); 4-amino-2-nitrotoluene (4A-2-NT); 2-amino-6-nitrotoluene (2A-6-NT); 1-amino-3-nitrobenzene (1A-3-NB); 3,5-dinitroaniline (3,5-DiNA); 2,4,6-trinitrotoluene (2,4,6-TNT); 1,3,5-trinitrobenzene (1,3,5,-TNB); 2-amino-4,6-dinitrotoluene (2A-4,6-DNT); 4-amino-2,6-dinitrotoluene (4A-2,6-DNT); Hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX); octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine (HMX);

1-acetylhexahydro-3,5-dinitro-1,3,5-triazine (TAX); and 1-acetyloctahydro-3,5,7-trinitro-1,3,5,7-tetrazocine (SEX).

RESULTS AND DISCUSSION

2,4,6-Trinitrotoluene and Corresponding Monoamines

From plots of NR uptake versus toxicant concentrations, the midpoint cytotoxicity value for 2,4,6-TNT was determined to be 0.033 mM. The corresponding amines, 2A-4,6-DNT and 4A-2,6-DNT, demonstrated cytotoxicities similar to each other (NR_{50} = 0.449 and 0.373 mM), but were at least ten times less toxic than the parent nitroaromatic compound.

2,4- and 2,6-Dinitrotoluene and Their Monoamines

From similar plots, 2,4-DNT (NR_{50} = 0.439 mM) was at least ten times less cytotoxic than 2,4,6-TNT in the assay. Midpoint cytotoxicities for 4A-2-NT and 2A-4-NT (NR_{50} = 0.269 and 0.328 mM) were only slightly lower than that of the 2,4-DNT parent compound.Likewise, 2,6-DNT (NR_{50} = 0.384 mM) was also significantly less toxic than 2,4,6-TNT in the assay. Midpoint cytotoxicity for the corresponding amine, 2A-6-NT (NR_{50} = 0.502 mM), occurred at a concentration only slightly higher than that of the 2,6-DNT parent.

1,3-Dinitrobenzene, 1,3,5-Trinitrobenzene, and Their Monoamines

Plots of NR uptake demonstrated that the most potent compound studied was 1,3,5-TNB (NR_{50} = 0.0099 mM). It was more than three times as cytotoxic as 2,4,6-TNT. A microbial metabolite of the compound, 3,5-DiNA (NR_{50} = 0.132 mM) showed considerably reduced midpoint cytotoxicity. A second nitrated benzene compound found in munitions wastestreams, 1,3-DNB (NR_{50} = 0.298) was far less toxic than 1,3,5-TNB in the assay. Its amine 1A-3-NB (NR_{50} = 1.1 mM) demonstrated considerably reduced cytotoxicity when compared with the parent compound.

Triazines and Tetrazocines

RDX and HMX and their acetylated derivatives, SEX and TAX, showed no significant cytotoxicity at any concentration after three separate assays for each compound. The highest concentrations applied to the system (100 mg/L) far exceeded the aqueous solubilities of the compounds.

Comparative Cytotoxicities with Two Other Screening Systems

As is shown in Table 1 for TNT and related nitroaromatic compounds, relative potencies in NR uptake assays are in the order TNB > TNT > DNB ≥ DNTs. Except for 2,6-DNT,which was somewhat more toxic than 1,3-DNB and 2,4-DNT in water fleas, toxicity orders are in general agreement. Correlation coefficients between NR_{50} values and LC_{50} values were 0.83 for water fleas and 0.91 for fathead minnows. For the corresponding amines in the table, with the exception of 3,5-DiNA and 1A-3-NB, NR_{50} values clustered from 0.27 to 0.5

Table 1 NR_{50} Values for Munitions and Related Compounds in H4IIE Cells and Their Acute Toxicities to Water Fleas and Fathead Minnows

Compounds	H4IIE NR_{50} (mM)	$(-1\sigma$ to $+1\sigma)$ (mM)	r	Water flea 48-hr LC_{50} (mM)	Fathead minnow 96-hr LC_{50} (mM)
2,4,6-TNT-associated nitroaromatics					
1,3,5-TNB	0.0099	(0.0083–0.012)	0.99	0.013	0.0052
				0.014[a]	0.0024[a]
2,4,6-TNT	0.033	(0.023–0.044)	0.99	0.052	0.013
1,3-DNB	0.298	(0.247–0.354)	0.98	0.295	0.042
				0.163[a]	0.1[a]
2,6-DNT	0.384	(0.316–0.453)	0.91	0.12	0.102
2,4-DNT	0.439	(0.408–0.472)	0.97	0.261	0.18
2,4,6-TNT-associated amino nitroaromatics					
3,5-DiNA	0.132	(0.101–0.164)	0.99	0.084	0.12
				0.075[a]	0.116[a]
4A-2-NT	0.269	(0.183–0.381)	0.98	0.09	0.16
2A-4-NT	0.328	(0.204–0.480)	0.98	0.15	0.45
4A-2,6-DNT	0.373	(0.298–0.458)	0.97	0.027	0.035
2A-4,6-DNT	0.449	(0.354–0.547)	0.96	0.023	0.077
2A-6-NT	0.502	(0.381–0.640)	0.96	0.093	0.33
1A-3-NB	1.1	(1.0–1.202)	0.96		
Triazines and tetrazocines					
RDX	None			None[b]	4–6 mg/L[c]
HMX	None				None[c]
SEX	None				
TAX	None				

Source: Data from Ref. 3, unless otherwise noted. [a]Ref. 7; [b]Ref. 4; [c]Ref. 5.

mM with overlapping ranges and direct correlation with LC_{50} values is not seen. Most monoamines were less toxic than the parent nitroaromatics in NR uptake and were less toxic than the parent nitroaromatics in NR uptake and fathead minnow assays, whereas the opposite was true with water fleas. None of the systems correlates with acute rat oral toxicities, for which the order is DNB $\gg$ DNTs $>$ TNB $>$ TNT, although the monoamines 2A-4-NT, 1A-3-NB, 2A-4,6-DNT are also less toxic in rats than corresponding nitro compounds. No significant cytotoxicity was noted for triazine- and tetrazocine-associated munitions in the assay. Although data are sparse, there appears to be little toxicity associated with RDX for *Ceriodaphnia dubia* and other invertebrates, and with HMX for fish, up to the limits of their solubilities [5]. In contrast, acute toxicity has been reported for RDX in fathead minnows [4].

CONCLUSIONS

All 2,4,6-TNT-associated munitions tested and their monoamines have responded measurably in NR uptake assays in H4IIE cells. The triazine and tetrazocine munitions, RDX and HMX, did not respond, nor did their acetylated congeners SEX and TAX. Responses to 2,4,6-TNT and associated nitroaromatic compounds were essentially consistent with two other systems used to screen munition samples, whereas the monoamines they form in the environment responded differently. Coupled to compatible extraction methods, NR uptake assays are potentially valuable in detecting 2,4,6-TNT and associated compounds or their mixtures in contaminated samples, whether or not they have been reduced in the environment. The same is not true of RDX- and HMX-associated munitions.

ACKNOWLEDGMENT

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